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Expression of SIBLINGs and Their Partner MMPs in Salivary Glands

ABSTRACT

Three members of the SIBLING family of integrin-binding phosphoglycoproteins (bone sialoprotein, BSP; osteopontin, OPN; and dentin matrix protein-1, DMP1) were recently shown to bind with high affinity (nM) and to activate 3 different matrix metalloproteinases (MMP-2, MMP-3, and MMP-9, respectively) *in vitro*. The current study was designed to document the possible biological relevance of the SIBLING-MMP activation pathway *in vivo* by showing that these 3 SIBLINGs and their known MMP partners are co-expressed in normal adult tissue. BSP, OPN, and DMP1 were invariably co-expressed with their partner MMPs in salivary glands of humans and mice. The 2 SIBLING proteins without known MMP partners, dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE), were also expressed in salivary glands. Expression of all SIBLINGs in this normal, non-mineralizing epithelial tissue suggests that they serve at least one function *in vivo* other than directly promoting matrix mineralization—a function we hypothesize involves local activation of MMPs.

KEY WORDS: SIBLING, MMP, bone sialoprotein, osteopontin, dentin matrix protein-1.

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INTRODUCTION

The SIBLING family (Small Integrin-Binding LIgand N-linked Glycoproteins) currently includes 5 members: BSP, OPN, DMP1, DSPP, and MEPE. The family is a conserved gene cluster with common exon-intron structures, the presence of the integrin-binding tripeptide, Arg-Gly-Asp (RGD), as well as conserved phosphorylation and N-glycosylation sites (Fisher *et al.*, 2001). The 4 acidic SIBLING proteins (BSP, DMP1, DSPP, and OPN) were discovered some years ago by several laboratories (Robey, 2002) in bones and teeth, while MEPE was discovered recently in association with tumors that cause phosphate wasting (Rowe *et al.*, 2000).

With the clear exception of osteopontin, the SIBLINGs are generally reported to be limited to bones and teeth in normal adults. OPN was reported in normal, non-mineralizing tissues, including: kidney (Shiraga *et al.*, 1992), lactating breast (Senger *et al.*, 1989), and immune cells (Patarca *et al.*, 1990). A 1992 report by Brown *et al.* noted the presence of OPN in several epithelial tissues, including salivary glands. In contrast, Kusafuka *et al.* (1999) noted that OPN was not expressed in salivary glands. BSP, originally found in bones and dentin (Fisher *et al.*, 1983), was reported to be expressed by trophoblasts (Bianco *et al.*, 1991) and ameloblasts (Chen *et al.*, 1998). DMP1 was initially thought to be dentin-specific (George *et al.*, 1995), but its mRNA has been found in bones (MacDougall *et al.*, 1998), the brain (Hirst *et al.*, 1997), and ameloblasts (George *et al.*, 1995). DSPP was also originally thought to be limited to dentin, but low levels of expression have been described in bones (Qin *et al.*, 2002) and ameloblasts (D'Souza *et al.*, 1997). The original publication of MEPE indicated, by RT-PCR, low levels in the normal adult brain and kidney in addition to higher levels in bones (Rowe *et al.*, 2000).

We have recently shown that BSP, DMP1, and OPN each binds with high affinity (nM) and specifically activates proMMP-2, proMMP-3, and proMMP-9, respectively. Furthermore, the SIBLINGs can specifically re-activate their corresponding TIMP-inhibited MMPs (Fedarko *et al.*, 2004), and BSP has been shown to enhance invasion *in vitro* by bridging MMP-2 to the cell surface *via* the $\alpha_v\beta_3$integrin (Karadag *et al.*, 2004). Thus, the expression of a SIBLING protein with or even near a source of its MMP partner can result in active proteases and local processing of proteins. It is not known if DSPP and MEPE also have MMP partners.

Our hypothesis is that documentation of the co-expression of SIBLINGs and their specific MMP partners in normal, non-mineralizing tissues will suggest that the SIBLINGs have functions other than direct control of mineralization.

MATERIALS & METHODS

Tissue Collection

Paraffin-embedded blocks of normal human parotid, submandibular, and minor salivary glands were obtained under IRB approval from the Cooperative Human

Table. Antisera and Riboprobes

Antisera

Antigen	ID	Description	Dilution	Type
Human BSP[1]	LFMb-25	carboxy-terminal region	10 μg/mL	mouse IgG_1
Human DSPP[1]	LFMb-21	DPP domain CSRGDASYNSDESKDNG	2.5 μg/mL	mouse IgG_{2b}
Human OPN[1]	LFMb-14	just C-terminal to RGD	2.5-10 μg/mL	mouse IgG_{2b}
Human MEPE	LFMb-33	within THRIQHNIDYLKHLSKVKKI	1:1 culture fluid	mouse IgG_1
Human MEPE[2]	LF-155	within last exon	1:200 dilution	rabbit polyclonal
Human DMP1[1,2]	LF-148	peptides (Jain *et al.*, 2002)	1:200 dilution	rabbit polyclonal
Human DMP1[1]	LFMb-31	epitope near RGD domain	10 μg/mL	mouse IgG_1
Human BSP[2]	LF-83	(Mintz *et al.*, 1993)	1:200 dilution	rabbit polyclonal
Mouse OPN	LF-175	bacterial recombinant	1:200 dilution	rabbit polyclonal
Mouse DSPP	LF-153	bacterial recombinant (DSP portion)	1:200 dilution	rabbit polyclonal
Human MMP-2	MAB13431	(Chemicon, Temecula, CA, USA)	10 μg/mL	mouse IgG_1
Human MMP-3[2]	MAB3306	(Chemicon)	10 μg/mL	mouse IgG_1
Human MMP-9	RB-1539	(Lab Vision, Fremont, CA, USA)	5 μg/mL	rabbit polyclonal

[1] Cross to monkey on paraffin sections.
[2] Cross to mouse on paraffin sections.

Riboprobes

Target	ID	Description	Insert	Plasmid	Reference
Human					
BSP	B65g	full-length coding cDNA	1.2 kbp	pBluescript	Fisher *et al.*, 1990
DMP1	hDMP1-E5-3	coding in last exon by PCR	1.4 kbp	pBluescript	
DSPP	hDSPP-1	DSP portion of DSPP exon 4 by PCR	0.7 kbp	pBluescript	
DSPP	hDPP510	(non-repeat) DPP of exon 4 by PCR	215 bp	pBluescript	
MEPE	hMEPEx45	full-length cDNA lacking exons 4&5	1.6 kbp	pBluescript	
OPN	OP10	full-length cDNA lacking exon 4	1.5 kbp	pBluescript	Young *et al.*, 1990
MMP-2	hMMP2	portion last exon by PCR	~ 320 bp	pBluescript	
MMP-3	hMMP3	portion last exon by PCR	~ 340 bp	pBluescript	
MMP-9	hMMP9	portion last exon by PCR	~ 320 bp	pBluescript	
Mouse					
BSP	mBSP1	full-length coding cDNA	~ 1 kbp	pCR II	Young *et al.*, 1994
DMP1	mDMP1	portion of last exon by PCR	~ 430 bp	pBluescript	
DSPP	mDSPP-2	DSP portion of exon 4 by PCR	~ 940 bp	pBluescript	
MEPE	mMEPE-493	portion of last exon by PCR	~ 380 bp	pBluescript	
OPN	mop3	full-length cDNA	~ 1 kbp	pCR II	Fisher *et al.*, 1995
MMP-2	mMMP2-514	portion 3′ flanking in last exon by PCR[10]	~ 400 bp	pBluescript	
MMP-3	mMMP3-489	portion 3′ flanking in last exon by PCR[11]	~ 240 bp	pBluescript	
MMP-9	mMMP9-491	portion 3′ flanking in last exon by PCR[12]	~ 560 bp	pBluescript	

Primers[a] used for PCR reactions (forward primer listed first):

hDMP1: ATTATAGAATT**CAAATGAAGACCCCAGTGACAGC** & TAATTAGGATCC**AATAGCCGTCTTGGCAGTC**
hDSP: GGCAGCCTCGAG**ACAGCAAATGGCATCCAGGG** & ATATATGGATC**CCTAATCTTGGCTCTTCCCAACAGC**
hDPP: ATAGAATTC**GATGATCCCAATAGCAGTGATG** & GAATGGA**TCCCATTGTTACCATTGCCATTACTG**
hMEPE: ATTATAGGTAC**CTCAAAGATGCGAGTTTTCTG** & CTGATGGGATCC**CTAGTCACCATCGCTCTCAC**
hMMP2: ATTAGGATCC**GGTCACAGCTACTTCTTCAAG** & ATATGGATC**CGCCTGGGAGGAGTACAG**
hMMP3: ATATGGATC**CAGCTGGCTTAATTGTTGAAAG** & TAATGGATC**CAACTGACAAATCGTCTTTATTA**
hMMP9: AATTGAATTC**AGAGAAAGCCTATTTCTGCCAG** & TAATGAATTC**GGTTAGAGAATCCAAGTTTATTAG**
mDMP1: ATGCGAATTC**GGCAAATAGTGACCACACGG** & GCATGG**ATCCCTTCATCATCGAACTC**
mDSPP-2: GGCAGCCTCGAG**AATGAGTTATCTATCAACAGC** & ATATATGGATC**CTACTGATCTTGGCTCTTCCCAG**
mMEPE-493: ATATG**AATTCAGCAGATGTCAATGATGC** & GGATGGA**TCCACTCATATGTGGGTCAATAACTTCAGC**
mMMP2: ATATTGGA**TCCCACGGGCCCTATCATCTTC** & TAATAGAAT**TCCACCTTGGCACACCTGTACC**
mMMP3-489: GCTTGGATCC**GAGATCCAAGGAAGGCATCCTG** & CGATGGAT**CCATCTACACAGTTCAGACAC**
mMMP9-491: **AACACGGATCCCCAACCTTTTCCAG** & CGATGG**ATCCTTGAAGGAAGAACCAACATTTCTGTCC**

[a] Bold letters are the complementary sequences within the template genomic DNA, and the remainder encode the restriction enzyme sites used for cloning and sufficient extra bases to ensure restriction enzyme digestion.

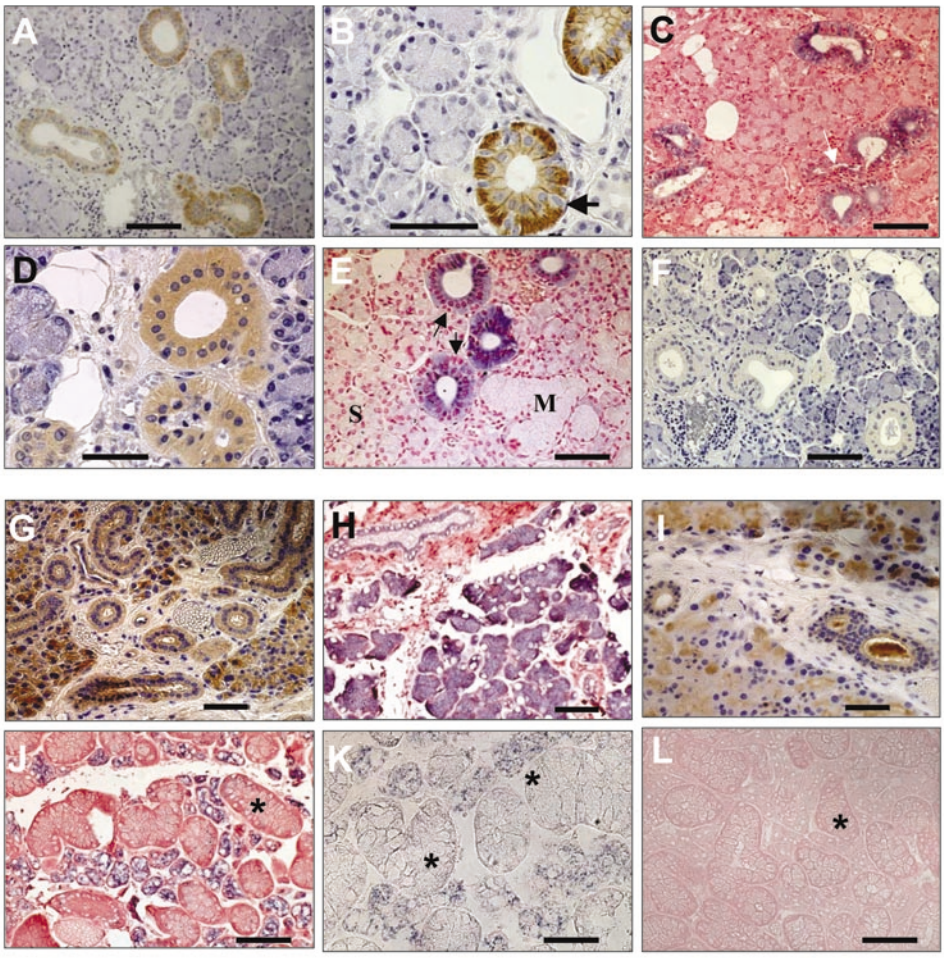

Figure 1. Co-expression of osteopontin and MMP-3 in human and mouse salivary glands. Immunolocalization (brown = 3,3′-diaminobenzidine, DAB; blue = hematoxylin counterstain) and *in situ* (blue stain, BCIP/NBT, with or without fast-red counterstain) in human (A-F) and mouse (G-L) paraffin sections. Staining of different but representative sections of human parotid ducts at low magnification **(A)** and high magnification **(B)** with OPN antibody (LFMb-14). Note the non-staining 'basal' cells (black arrow). Panel **(F)** is a representative IgG control. OPN antisense staining of human submandibular ducts **(C).** All human ducts are also positive with antibody **(D)** and antisense **(E)** to MMP-3. Note that MMP-3 message is weak or absent from the 'basal' cells of the striated ducts (panel E, black arrows). In contrast, female mouse parotid **(G)** and submandibular glands **(I)** stain positive for OPN protein (LF-175) in all ducts as well as parotid serous acini and submandibular seromucous acini, respectively. The message for OPN is also seen in the ducts and serous acini of the female mouse parotid **(H)**. In the mature male mouse, submandibular gland ducts (except for the granular convoluted tubules, GCT noted as *) and the seromucous acini **(J)** stain for OPN message. Mouse MMP-3 message is similarly distributed in the submandibular ducts and seromucous acini of both sexes, except for the lack of staining in the GCT (male shown, **K**). Panel **(L)** is a representative sense strand control. M and S labels represent typical mucous and serous acini, respectively, of human glands. White arrows indicate intercalated ducts. Treatment with proteinase K during *in situ* preparation destroys the fast-red counterstaining properties of mouse nuclei. Bar: 50 μm.

Tissue Network (Charlottesville, VA, USA). Fresh parotid, submandibular, and sublingual salivary glands of monkeys (*M. nemestrina*) were obtained as surgical waste at autopsy from the Tissue Distribution Program, National Primate Research Center (University of Washington, Seattle, WA, USA). Mouse salivary glands were obtained as surgical waste from normal mice previously culled from a breeding pool. After a 24-hour fixation in neutral-buffered 4% paraformaldehyde, tissues were paraffin-embedded and sectioned.

Antibody Production

The monoclonal and polyclonal antisera used for this study are listed (Table). We made new antibodies using standard mouse hybridoma technology (Maine Biotechnology Services, Portland, ME, USA) and in New Zealand white rabbits (Covance, Denver, PA, USA) using approved animal care protocols at the contract facilities. The antigens were as follows: LFMb-25, human BSP amino acids 159-317 (with additional cysteine at the amino terminus for conjugation to maleimide-activated keyhole limpet hemocyanin, KLH; Pierce, Rockford, IL, USA) was made as a His_6-fusion protein in *E. coli* and purified on His-Bind resin (Novagen, Madison, WI, USA). Full-length mouse OPN (LF-175), human OPN (LFMb-14) (amino acids 159-300), and mouse DSPP (LF-153, amino acids 49-363) were made as His_6-fusion proteins in bacteria, purified, and directly used as antigens. Monoclonals to human MEPE (LFMb-33, amino acids 42-525) and DMP1 (LFMb-31, amino acids 62-513) His_6-fusion proteins were conjugated through amino groups to EDC-activated KLH (Pierce). All synthetic peptides were conjugated to maleimide-KLH. MMP antibodies were from commercial sources (Table).

Immunohistochemistry

Standard immunoperoxidase techniques involved Histostain-SP or PolyPlus-peroxidase detection systems (Zymed, San Francisco, CA, USA). Each section was incubated for 1 hr at room temperature with the primary antibody (Table). Negative controls included non-immune serum for rabbit antisera and IgG-isotype controls for monoclonals.

Preparation and Detection of RNA Probes

A summary of the sense and antisense riboprobes used for human and mouse SIBLINGs and MMPs is shown (Table). Human SIBLING probes were used on monkey sections. Full-length human MEPE cDNA was made by PCR with the use of a brain cDNA template (Multiple Tissue cDNA Panel, #1420-1, BD Bioscience, Palo Alto, CA, USA). The final product was cloned into pBluescript-KS (Stratagene, La Jolla, CA, USA). For all other PCR-based probes, the purified PCR products were digested with the appropriate restriction enzymes engineered into the oligonucleotides and cloned into PCR-II (mop-3 and mBSP1, Invitrogen, Carlsbad, CA, USA) or pBluescript-SK. PCR oligonucleotides are listed (Table). Clones were verified by DNA sequencing.

Both sense and antisense probes were labeled with use of the Digoxigenin (DIG) RNA-Labeling Mix (Roche, Mannheim, Germany) and the appropriate RNA-polymerases. The amount of DIG-labeled control (sense) probe used was always greater than that used for the antisense (as determined by comparison of serial-dilution dot blots on charged nylon membranes). *In situ* detection was by the InnoGenex™ Universal-ISH-BCIP/NBT Kit (Innogenex, San Ramon, CA, USA).

RESULTS

For the *in vitro* binding and activation of specific MMPs by SIBLINGs (Fedarko *et al.*, 2004) to be biologically significant, the pairs must be co-expressed in the same cell or within a short distance of each other. The expression patterns of the 5 SIBLINGs and 3 MMPs in human salivary glands were determined by immunohistochemistry and verified by *in situ* hybridization. Monkey major glands gave the same results as the human glands for all 8 gene products (data not shown). All 5 SIBLINGs were similarly identified in mouse glands, but the MMP localizations were by *in situ* identification only.

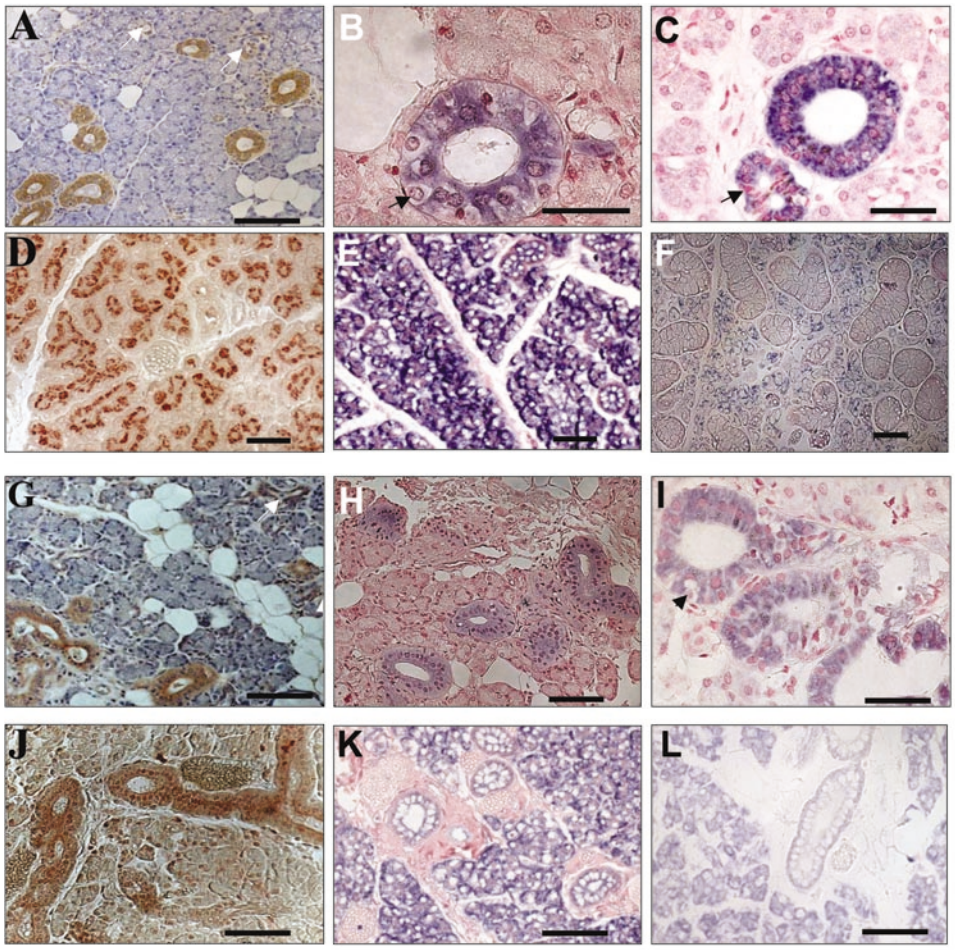

Figure 2. Co-expression of BSP with MMP-2 as well as DMP1 with MMP-9 in human and mouse salivary glands. Representative staining of BSP protein (LFMb-25, panel **A**) and message **(B)** in human parotid and submandibular glands, respectively. MMP-2 message is also expressed in human submandibular gland ducts **(C)**. A similar distribution is seen for human DMP1 protein (LFMb-31) **(G)** and message **(H)**, as well as for MMP-9 message **(I)** in submandibular ducts. In contrast to primates, mouse BSP protein (LF-83, **D**) and message **(E)** in the parotid as well as DMP1 protein (LF-148, **J**) and message **(K)** in representative female submandibular glands show that these SIBLINGs are expressed in both the non-GCT portions of all ducts and in the seromucous acini. Mouse MMP-2 message **(F)** and MMP-9 message **(L)** are also expressed in both non-GCT ducts and seromucous acini. White arrows indicate intercalated ducts, and black arrow indicate 'basal' cells. Treatment with proteinase K during *in situ* preparation destroys the fast-red counterstaining properties of mouse nuclei. Bar: 50 μm.

Osteopontin and its partner MMP-3 were co-localized at both the protein and mRNA levels in primates and mouse salivary glands. In humans, OPN (Figs. 1A-1C) and MMP-3 (Figs. 1D, 1E) were present throughout the entire ductal system (intercalated, striated, excretory ducts), as shown by immunohistochemistry and *in situ* hybridization, but were absent in both serous and mucous acini. Within the striated ducts, OPN immunoreactivity was distinctly more prominent in the basal half, in direct association with the striations and cell junctions (Fig. 1B). Occasional cells within the striated ducts that were devoid of both striations and contact with lumen (sometimes known as basal cells) appeared free of both OPN protein and message (Figs. 1B, 1E, black arrows), suggesting that only mature ductal cells express the 2 proteins. Like the primates, ductal segments of mouse major glands expressed OPN (Figs. 1G-1J) and MMP-3 (Fig. 1K), except for the unique granular convoluted tubule (GCT, noted by * in several panels), which did not express either gene product (Figs. 1J, 1K). Although mouse OPN was often associated with the distinctive basal striations, this pattern was less prominent than in the primates. In contrast to primates, however, mouse OPN (Figs. 1G-1J) and MMP-3 (Fig. 1K) also were observed in the seromucous acini of the submandibular gland and serous acini of the parotid gland. Controls for both immunostaining and *in situ* were uniformly negative and are represented by IgG control (Fig. 1F) and sense strand (Fig. 1L), respectively.

The staining patterns for the other 4 SIBLINGs—BSP (Figs. 2A, 2B), DMP1 (Figs. 2G, 2H), DSPP (DSP domain, Figs. 3A, 3B, with DPP domain not shown), and MEPE (Figs. 3C, 3D)—in human salivary glands by both antisera and *in situ* were the same as that for OPN. They were expressed throughout the length of the duct systems with uniformly negative acini. As was observed with OPN, occasional primate 'basal' cells located within a duct were negative for both protein and mRNA for these 4 SIBLINGs (*e.g.*, Fig. 2B, black arrow). However, unlike the basal distribution of OPN, all other SIBLINGs exhibited a

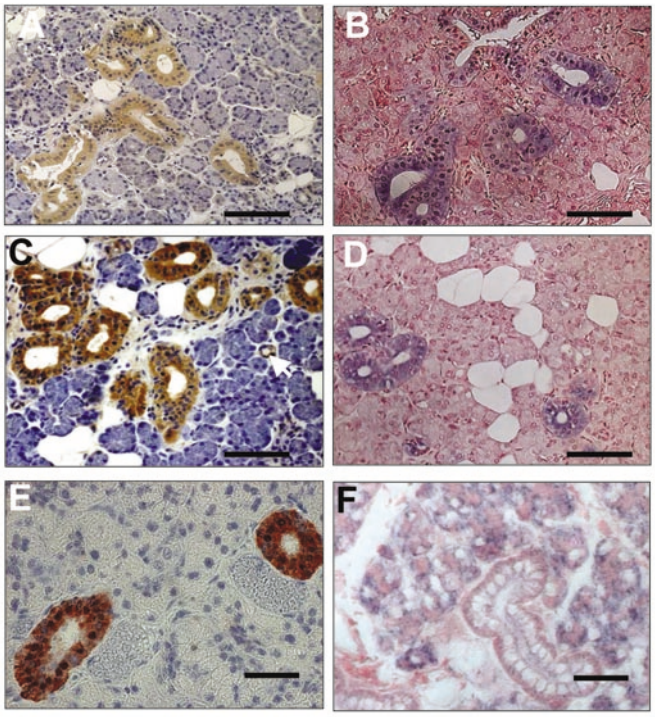

Figure 3. DSPP and MEPE are expressed in human and mouse salivary glands. Representative staining of DSPP protein (panel **A**, DPP portion, LFMb-21) in human parotid and message (panel **B**, DSP portion) in human submandibular ducts. A similar limited distribution is seen for human MEPE protein in the parotid ducts (panel **C**, LFMb-33) and message **(D)** in submandibular ducts. As was the case for OPN, BSP, and DMP1, male mouse submandibular glands are positive for MEPE message **(F)** in both seromucous acini and non-GCT ducts. The DSPP protein is located only in the ducts in the female mouse submandibular gland **(E)**, when an antibody to the DSP domain (LF-153) is used. White arrows indicate intercalated ducts. Treatment with proteinase K during *in situ* preparation destroys the fast-red counterstaining properties of mouse nuclei. Bar: 50 μm.

more even basal-luminal distribution within the striated duct cells. MMP-2 and MMP-9 co-localized with their partner SIBLINGs, BSP and DMP1, respectively, in each gland, including a general lack of staining in the 'basal' cells (*in situ* data are shown; Figs. 2C, 2I). Most of the sections did not have obvious duct luminal contents; however, when present, they tended to stain positive for the SIBLINGs, suggesting at least some secretion into the lumen (data not shown).

The immunohistochemistry and *in situ* patterns of both mouse BSP (Figs. 2D, 2E) and DMP1 (Figs. 2J, 2K), as well as MEPE *in situ* (Fig. 3F), mirrored that of mouse OPN (see Figs. 1G, 1H). They were present in both the non-GCT ductal elements and seromucous acini. Interestingly, mouse DSPP immunolocalization (Fig. 3E) was more like that of primate SIBLINGs, being found only in the ductal system (non-GCT) and not in the acini. In the mouse, the MMPs continued always to co-localize with their SIBLING partners—MMP-2 (Fig. 2F) with BSP, MMP-3 (Fig. 1K) with OPN, and MMP-9 (Fig. 2L) with DMP1. As in the primates, the mouse MMPs were observed only in cells that also expressed their partner SIBLING. Although not shown, human minor salivary glands had the same staining patterns as the major glands for all 8 gene products.

DISCUSSION

The purpose of this study was to verify that both the SIBLINGs and their MMP partners are co-expressed by cells of salivary glands and therefore can be expected to form spontaneously *in vivo* the same high-affinity (nM) complexes observed *in vitro*. Also included were the 2 members of the SIBLING family whose MMP partners, if any, are currently unknown.

Our results confirm those of Brown *et al.* (1992), who showed OPN expression in ductal elements of human salivary glands, although, in all 3 species, we saw intense basal staining patterns in the striated ducts rather than the luminal pattern suggested by those authors. The expressions of BSP, DMP1, DSPP, and MEPE were essentially uniform throughout the cells of the entire ductal system of primate salivary glands. The same expression pattern within the ducts was seen for mice, except that none of the SIBLINGs was expressed in the male mouse's granular convoluted tubule (GCT) segments. This prominent segment of the rodent submandibular duct system is a unique, androgen-dependent structure and is known to produce several bioactive compounds (Pinkstaff, 1998). Primates do not have an equivalent of the GCT within their salivary duct system. Although the overall intensity of duct cell immunoreactivity and mRNA signals varied slightly among the SIBLINGs, no consistent differences between segments of the ductal system (intercalated, striated, and interlobular) were apparent in the primate glands. What is particularly interesting is that in mice, but not in primates, all of the SIBLINGs except DSPP were also expressed in the serous acini of the parotid and the seromucous acini of the submandibular glands.

In all 3 species, MMP-2, MMP-3, and MMP-9 were shown to co-localize in the mature ductal cells with their partner SIBLINGs (BSP, OPN, and DMP1, respectively). The mouse salivary glands were particularly revealing in this respect, because, like the SIBLINGs, the messages for the 3 MMPs were invariably missing in the GCT of the male mouse ductal system. Furthermore, these 3 SIBLINGs and their partner MMP mRNAs were uniquely found in the acini of the rodent. These observations provide convincing evidence that the SIBLING-MMP pairing described *in vitro* may also be important *in vivo*. Recently, others have independently verified, by immunohistochemistry, that MMP-2 and MMP-9 (MMP-3 was not reported) are expressed in the ducts, but not the acini, of normal human salivary glands (Nagel *et al.*, 2004). Interestingly, they also showed the same expression pattern of the tissue inhibitors of matrix metalloproteinases, TIMPs. Previously, we have shown *in vitro* that the appropriate SIBLING partner will re-activate a TIMP-inactivated MMP upon binding (Fedarko *et al.*, 2004), thereby suggesting that the MMPs may remain active in the ducts/saliva, even in the presence of these inhibitors.

Numerous earlier reports have shown the up-regulation of the SIBLINGs in several different tumors (Bellahcéne *et al.*, 1996; Waltregny *et al.*, 1998; Chaplet *et al.*, 2003). Because most aggressive tumors are associated with the up-regulation of MMP-2, MMP-3, and/or MMP-9, the SIBLING-MMP complexes may aid the metastatic processes of tumor cells. Indeed, we have recently shown that BSP enhances the invasion potential of many different cancer cell lines by bridging MMP2 to the $\alpha_v\beta_3$ integrin (Karadag *et al.*,

2004). Within growing bones and teeth, these same complexes could logically be hypothesized to be involved in the processing of proteins, thereby aiding the maturation of the matrix prior to mineralization. However, because the ductal cells in adult salivary glands are immobile and unlikely to be degrading connective tissue (or basement membranes), as would metastatic cancer cells, it is possible that the SIBLING/MMP complexes are involved in the normal turnover of cell-surface and/or pericellular matrix proteins damaged by oxidative by-products of these metabolically active cells. Indeed, since osteoblasts and odontoblasts are cells with an extremely high metabolism, they may also require proteolytic activity to maintain their local micro-environment. Finally, because MMPs are known to be secreted in the saliva (Wu *et al.*, 1997), it is possible that specific salivary proteins are processed by these complexes as they move down the ducts and/or within the environment of the mouth.

In summary, all SIBLINGs and their MMP partners were co-expressed in the salivary ducts of all 3 species, except the GCT ducts of male mice. In mice, all of the SIBLINGs except DSPP were also expressed in the serous acini of the parotid and seromucous acini of the submandibular gland. Due to the high (nM) affinity and specificity of the interactions between the SIBLING-MMP partners (Fedarko *et al.*, 2004), only the correct complexes will likely form upon co-secretion, even though all of the proteins are present. Three future challenges include: (1) finding possible MMP partners for DSPP and MEPE; (2) identifying the specific protein substrates of the SIBLING-MMP complexes; and (3) determining the balance between inhibition by TIMPs and activation by SIBLINGs in different tissues.

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